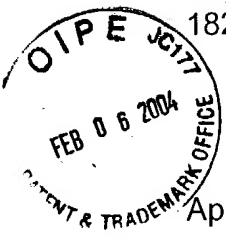


Atty. Docket No.
182.0010/WRAIR00-26

PATENT



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): ALVING et al

Serial No.: 09/859,389

Examiner: Cheu

Filed: May 18, 2000

Art Unit 1641

Title: DETECTION OF ANTIBODIES TO SQUALENE IN SERUM

DECLARATION UNDER 37 C.F.R. §1.132

1. I, Dr. Carl R. Alving, am a joint inventor of the invention subject to this patent application and make this declaration based upon personal knowledge in support of the patentability thereof.

2. I have reviewed and analyzed the primary reference relied upon by the Examiner in rejecting the claims of the application Asa et al US 6,214,566 (hereafter "the Asa Patent").

3. The technical merit and hypothesis suggested by Asa in the patent and collateral papers has been vigorously criticized and rejected by the scientific community as evidenced by my own published "Letters to the Editor" appearing in *Experimental and Molecular Pathology*, **68** 196-198 (2000) (a true reprint copy attached hereto under Tab 1) and the subsequent report by the *Institute of Medicine* (a true copy attached hereto under Tab 2). Those documents detail the flawed scientific techniques used and conclusions reached by Asa.

4. While the Asa patent reports the existence of an assay for antibodies to squalene, it is non-specific to other antigenic epitopes (See Col. 7 lines 1-6). The Asa

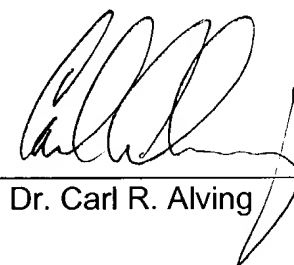
patent is not enabling of the invention disclosed and claimed in our patent application. Asa does not teach or suggest the identification of an antibody specific to squalene and/or an antibody exhibiting strong dose dependant binding to squalene. That is, Asa does not teach or enable a method for detecting antibodies to squalene that have little or no cross reactivity to squalane as admitted in Asa's patent at Column 7 and also does not enable or identify monoclonal antibodies specific to squalene.

5. Your Declarant says nothing further.

VERIFICATION

I hereby declare that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Date: 2/2/04



Dr. Carl R. Alving

LETTERS TO THE EDITOR

To the Editor:

A recent article in this journal by Asa *et al.* (2000) purports to measure serum antibodies to squalene. The paper fails to establish the validity of the test. The essential flaws involve selection of proper positive controls and proper negative controls, quantitative methods, and selection of study populations.

The authors hypothesize that antibodies are induced by "the adjuvancy of squalene," such that injection of squalene could elicit antibodies to squalene. One approach might be to inject squalene into an experimental animal to determine *first* whether the injection can induce the purported antibodies and *second* whether the assay can detect the induced antibodies. Antibodies induced by injection, if they exist, could then serve as a positive control for the unvalidated assay.

The assay describes no positive controls that actually validate the assertion of detecting antibodies to squalene. Such positive controls would consist of comparable serum samples demonstrated to contain anti-squalene antibodies after injection with squalene.

The authors assert that they have positive controls, in the form of two human subjects previously injected with a squalene-containing placebo during a clinical trial at the National Institutes of Health. However, the authors provide no preinjection results to establish that intentional injection of squalene led to antibodies to a substance already present in the body.

The assay also lacks elementary negative controls routinely run in enzyme-linked immunoassays. Such negative controls are required to prove that the assay is not detecting cross-reacting substances. In a new, unproven assay that claims to detect a novel antibody, one must prove specificity. There were no negative controls in which the human serum containing the presumed antibodies was omitted or in which the avidin-conjugated horseradish peroxidase was omitted. There is no evidence that the assay was not simply measuring other IgG molecules with nonspecific binding to squalene. This could be easily accomplished by substituting an oil

molecule similar to squalene. An excellent negative control would be squalane, the fully hydrogenated form of squalene.

The unknown human serum samples were tested only at a single dilution (1:400). Most assays for naturally occurring antibodies, particularly antibodies to lipids, start at a higher concentration of serum, typically a dilution of 1:50. Thus, the method of Asa *et al.* could miss the presence of antibodies detectable at a higher concentration of serum. It is possible that normal blood donors could give positive results at a higher concentration of serum.

A further drawback of using only a single dilution of serum, rather than a series of dilutions, is that there is no way to obtain a quantitative measure of the degree of activity in the sample. Titers are routinely obtained when antibody levels are measured. The absence of quantitation in this assay weakens meaningful comparisons between unknown serum samples from subjects accrued in a nonrandom manner.

Figure 1, said to show "antisqualene antibody responses," is particularly flawed. In this figure, unspecified quantities of squalene were added as aqueous dilutions of 1:10, 1:100, 1:1000 and 1:10,000 for impregnation of nitrocellulose. No explanation is provided for how an oil such as squalene, not soluble in water, could be diluted in water by the published methods. Further, a washing solution containing polyoxyethylene sorbitan monolaurate could have detergent-like qualities that could remove squalene. Despite the extensive dilutions of the squalene, there is no evidence of a dilution curve (assessing each strip vertically), regardless of whether the antibody reactions were rated as 3+, 2+, or 1+. This suggests that nonspecific binding of serum immunoglobulin may have occurred.

The conclusions of Asa and colleagues, purporting to correlate anti-squalene with Gulf War illnesses, in our opinion, rely on circular logic. Positive results with an assay not previously validated to detect antibodies cannot be used as scientific proof that antibodies to the antigen exist in samples of unknowns. It is premature to proceed directly to testing

serum samples from healthy people and sick people before conducting the fundamental validation steps.

The critique offered here is not meant to imply that antibodies to squalene do not or cannot exist. As pointed out by the authors, extensive work demonstrates that antibodies to cholesterol, a molecule for which squalene serves as a precursor, are found in virtually all normal human sera. A recent report proposes that naturally occurring antibodies to cholesterol may serve a vital physiologic function in helping regulate low-density lipoprotein metabolism in humans (Alving and Wassef, 1999).

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GULF WAR and HEALTH

VOLUME 1

Depleted Uranium, Pyridostigmine Bromide, Sarin, Vaccines

Carolyn E. Fulco, Catharyn T. Liverman, Harold C. Sox, *Editors*

Committee on Health Effects Associated with Exposures During the Gulf War

Division of Health Promotion and Disease Prevention

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Contents

Front Matter -- [PDF file](#)

Executive Summary -- [PDF file](#)

1 Introduction -- [PDF file](#)

2 Illnesses in Gulf War Veterans -- [PDF file](#)

3 Methodology -- [PDF file](#)

4 Depleted Uranium -- [PDF file](#)

5 Sarin -- [PDF file](#)

6 Pyridostigmine Bromide -- [PDF file](#)

7 Vaccines -- [PDF file](#)

CONTENTS

ISSUES IN IDENTIFYING ADVERSE EFFECTS	268
Surveillance, 269	
Difficulties in Detecting Adverse Events Due to Vaccination	
in Humans, 270	
Difficulties in Detecting Adverse Events Due to Vaccination	
in Animals, 271	
ANTHRAX VACCINE.....	272
Toxicology, 275	
Human Studies, 280	
BOTULINUM TOXOID	287
Toxicology, 289	
Human Studies, 291	
MULTIPLE VACCINATIONS	294
Toxicology, 296	
Human Studies, 299	
SQUALENE	307
Dietary Intake, Absorption, Distribution, and Metabolism, 307	
Animal Studies, 308	
Use of Squalene as a Vaccine Adjuvant, 309	
Gulf War Issues, 311	
Future Research Directions Regarding Squalene, 312	
CONCLUSIONS.....	312
Anthrax Vaccine, 312	
Botulinum Toxoid, 313	
Multiple Vaccinations, 314	
REFERENCES.....	314

SQUALENE

The committee was asked to examine the literature on potential health effects of squalene since it has been raised as an issue of concern to Gulf War veterans. A recent GAO (1999a) report found that at the time of the Gulf War, DoD had concerns about having a sufficient quantity of the anthrax vaccine and sufficient time to fully immunize the troops (GAO, 1999a). However, DoD has stated that it decided against the use of novel adjuvant formulations (e.g., formulations with squalene) because of lengthy FDA relicensure requirements (GAO, 1999a). The following section provides a brief overview of the animal and human studies that have been conducted and concludes with the committee's thoughts on directions for future research. The committee was not asked to draw conclusions on the strength of the evidence for an association between exposure to squalene and adverse health effects.

Squalene¹⁴ is a polyunsaturated terpene hydrocarbon that is widely distributed in nature. It is found in human sebum (a skin surface lipid) and is a precursor in the synthesis of human cholesterol (Final Report, 1982; Kelly, 1999). Its name stems from its abundance in shark (*Squalus* spp.) liver oil,¹⁵ from which it was first isolated (Liu et al., 1976). Squalene also is found in other fish oils, olive oil (0.7 percent), wheat germ oil, rice bran oil, and many other foods.

For more than 25 years, squalene has been used commercially as an emollient for topical application of more than 300 cosmetic formulations, including suntan preparations, bath oil, eye makeup, hair preparations, lipstick, baby powder, and skin care preparations. Cosmetic concentrations range from less than 0.1 to more than 50 percent. Squalene also is available as a dietary supplement; as a constituent of certain pharmaceuticals, including suppositories; and as a carrier of lipid-soluble drugs (Final Report, 1982; Kelly, 1999). As described below, squalene is being investigated for a number of potential medical purposes.

Dietary Intake, Absorption, Distribution, and Metabolism

In the 1970s, the average dietary intake of squalene in the United States was calculated at 24 mg per day (given a daily dietary intake of 2,000 calories) (Liu et al., 1976). Olive oil is particularly rich in squalene. Among Asians, shark liver oil supplements containing squalene are popular over-the-counter folk remedies (Asnis et al., 1993). The average total squalene exposure of humans from all routes of administration does not appear to have been studied. In case studies, excessive ingestion of squalene from dietary supplements has led to lipoid pneumonia (Asnis et al., 1993).

¹⁴Its chemical formula is 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene.

¹⁵Squalene concentrations in shark liver oil range between 50 and 80 percent (Liu et al., 1976).

Squalene is absorbed through several routes of administration, depending on the species (Final Report, 1982). In mice, squalene penetrates slowly and poorly through the skin at a rate of 0.12 nmol/cm² per minute (Final Report, 1982). Subcutaneous administration in rabbits leads to increases of stored squalene in liver, muscle, and skin (Final Report, 1982). Virtually all squalene administered orally to rats (96–100 percent) is unabsorbed (Albro and Thomas, 1970).

In humans, about 60 percent of dietary squalene is absorbed through the gastrointestinal tract, with the remainder excreted in feces (Strandberg et al., 1990). A significant fraction of absorbed squalene is converted into cholesterol. Squalene is distributed throughout human tissues, with greatest concentrations in skin and fat (Kelly, 1999). Squalene in human serum comes from endogenous cholesterol synthesis and from diet (Strandberg et al., 1990; Kelly, 1999). Peak serum levels are attained 9–12 hours after ingestion (Gylling and Miettinen, 1994).

Animal Studies

There are few published studies of squalene toxicity in animals or humans (Kelly, 1999). Kamimura and colleagues (1989) examined subacute toxicity in dogs after a single oral squalene dose of 1,200 mg/kg. Over the next 3 months, accumulation was noted in several tissues, especially liver, but there were no signs of toxicity based on testing of serum and liver function. In contrast to humans, who absorb 60 percent of ingested squalene, this study reported that dogs absorb a relatively small percentage and excrete most in feces (83 percent). Thus, the relevance of this study to humans is unclear.

Squalene's toxicity is considered low, with an oral LD₅₀ (median lethal dose) in mice at greater than 50 ml/kg (Final Report, 1982). No toxic responses were noted after subcutaneous and intramuscular injections of 0.5 ml per 20g mouse (25 ml/kg) of squalene (C₃₀H₆₂), a saturated and more stable version of squalene.

In a neuropathology study, squalene was administered subcutaneously to 10 rats (and 5 control rats) at a dose of 20 g/kg body weight for 4 consecutive days (Gajkowska et al., 1999). The rats' peripheral and central nervous systems were examined via electron microscopy 7 or 30 days from initiation of the experiment. After 7 days, disturbances in the myelin sheath were observed; these disturbances were more pronounced at 30 days. There was some swelling of Schwann cells in the peripheral nervous system. Fibroblasts were activated and showed signs of increased collagen production. In the central nervous system, squalene triggered swelling of astrocytes in white matter and in the hippocampus, especially near blood vessels. Lipid droplets accumulated in myelin in both the central and the peripheral nervous systems.

The pertinence of this neuropathology study is difficult to gauge because the dose was extremely high and the report provided minimal information about the study's methodology (especially handling of controls). Additionally, it is not uncommon to detect occasional astrocytic or neuronal swelling or mitochondrial swelling in electron micrographs of normal tissue. Further, damage appeared to be localized, not global, targeting, for example, a single myelin fiber or axon.

Squalene has attracted the interest of arthritis researchers because of its ability to activate the immune system nonspecifically. It was one of the constituents used in the 1970s to create the first animal models of multiple sclerosis, known as experimental allergic encephalomyelitis (EAE) (Beck et al., 1976). Squalene is one of several adjuvants (such as incomplete Freund's adjuvant) found to induce arthritis in *susceptible* rat strains and has been used in the generation of animal models of arthritis (Whitehouse et al., 1974; Lorentzen, 1999). The effect is so pronounced that researchers have coined the term "squalene-induced arthritis." After a single intraarticular injection of 50 μ L squalene into Lewis (Yoshino, 1996) and Dark Agouti rats (Yoshino and Yoshino, 1994), animals experienced moderate joint inflammation by day 6, followed by more severe chronic arthritis by day 21. The inflammation was marked by joint swelling and infiltration of CD5⁺ and $\alpha\beta$ ⁺ T cells. Similarly, intradermal injection of 200 μ L squalene into Dark Agouti rats produced arthritis (Lorentzen, 1999). Although the mechanisms are not fully understood, the inflammation is blocked by agents that suppress T cells (Yoshino, 1996; Sverdrup et al., 1998). Animal studies do not report whether injection of squalene produces antisqualene antibodies.

In summary, there is limited published information about squalene toxicity. The human relevance of what has been published is unclear because of species differences in absorption. Squalene has been found to produce arthritis and neuropathology under select conditions in animals; the relevance to humans of these toxicity findings is uncertain.

Use of Squalene as a Vaccine Adjuvant

Squalene is currently being studied for a number of medical purposes including treatment of hypercholesterolemia (Chan et al., 1996); as an antidote to reduce the toxicity of accidentally ingested drugs (Kelly, 1999); and as an adjunctive therapy in cancer treatment to potentiate the cytotoxic activity of some chemotherapeutic agents (Kelly, 1999). The area of research that is of particular relevance to this chapter is the use of squalene as a vaccine adjuvant or as a component of a vaccine adjuvant.

The dose of an adjuvant is typically small (in the microgram range), and the route of administration is usually intramuscular. Squalene has been tested primarily as one component of the vaccine adjuvant MF59. MF59 is an oil-in-water microemulsion, consisting of squalene, polysorbate 80 (Tween 80, polyoxyethylene sorbitan monooleate), and sorbitan trioleate (Graham et al., 1996). FDA has not yet approved any experimental vaccines with squalene-containing adjuvants.

The safety and efficacy of MF59 has been tested in a number of animal species with recombinant and natural antigens. Both short-term (approximately 2 weeks) and long-term (8 months) studies have been conducted and have detected some minor and transient changes in clinical laboratory parameters and histopathology (Ott et al., 1995). As described earlier in this chapter, a study by Ivins and colleagues (1995) on numerous combinations of adjuvants with the

purified anthrax protective antigen found adverse effects from one of the adjuvant combinations containing squalene; other adjuvants containing squalene did not elicit adverse reactions. Tests of an HIV candidate vaccine with MF59 found no embryotoxic or teratogenic effects in dogs or rabbits (Ott et al., 1995).

Clinical studies of MF59 and other squalene-containing adjuvants have been conducted with candidate malaria, HSV (herpes simplex virus), HIV (human immunodeficiency virus), and influenza vaccines (Ott et al., 1995; GAO, 1999a). Study populations for the clinical trials have included adults, elderly, and children and infants (Ott et al., 1995).

HIV Vaccine Trials

Keefer and colleagues (1996) investigated the safety and immunogenicity of a candidate HIV-1 vaccine in combination with MF59, with or without an additional immune modulator, MTP-PE (muramyl tripeptide linked covalently with dipalmitoyl phosphatidylethanolamine). Vaccination with the candidate vaccine Env 2-3 in MTP-PE/MF59 was associated with significant adverse effects; severe, though short-lived, systemic and/or local reactions occurred in 15 of 30 vaccinees. In contrast, Env 2-3 in MF59 without MTP-PE was relatively well tolerated; severe local and/or systemic reactions occurred in only 2 of 18 subjects. There were no severe reactions in the eight subjects that received MF59 alone.

Graham and colleagues (1996) evaluated the safety and immunogenicity of another candidate vaccine for HIV, the recombinant glycoprotein 120, formulated with MF59 with or without MTP-PE. Vaccines that contained MTP-PE caused a greater number of moderate or severe local and systemic reactions (of 16 participants, 4 had local reactions and 13 had systemic reactions) than did vaccine formulated with MF59 alone. Of 16 vaccinees, 7 had local reactions and 0 had systemic reactions.

The National Institute of Allergy and Infectious Diseases (NIAID)-sponsored AIDS Vaccine Evaluation Group examined safety data from 1,398 HIV-negative, healthy volunteers who were enrolled in 25 multicenter, randomized double-blind studies evaluating 11 HIV candidate vaccines (Keefer et al., 1996). The study examined the adverse effects of a number of adjuvants, including MF59 and MF59 formulated with the biological response modifier MTP-PE. MTP-PE was associated with moderate to severe local reactions as well as with self-limited severe systemic reactions that resolved within 2-3 days. The same vaccines in the MF59 emulsion alone were well tolerated (Keefer et al., 1996).

Influenza Vaccine Trials

The safety and efficacy of MF59 have been evaluated in pilot studies (Keitel et al., 1993) and clinical trials (Martin, 1997; Menegon et al., 1999; Minutello et al., 1999). A study by Martin (1997) assembled data from eight randomized controlled clinical trials over four influenza seasons; 984 elderly

volunteers (older than 65 years) received the adjuvanted vaccine, and 823 elderly volunteers received a conventional influenza vaccine. More than 20 percent of the volunteers who received the adjuvanted vaccine had local reactions. Myalgia was the only systemic effect to have been significantly more common in those receiving the vaccine with a squalene-containing adjuvant (3.9 percent) than those receiving the vaccine without the adjuvant (1.8 percent). All adverse events were recorded for 1 week after vaccination. Hospitalization and mortality were followed during the influenza season. The group receiving the adjuvanted vaccine had similar hospitalization rates and lower mortality than subjects receiving the conventional vaccine.

To date, clinical studies of the MF59 adjuvant that contains squalene have not shown any adverse health effects beyond transient acute effects.

Gulf War Issues

A recent study by Asa and colleagues (2000) reports on the development of an anti-squalene antibody assay to detect antibodies to squalene in the circulation. Blood samples from 144 Gulf War era veterans or military employees, 48 blood donors, 40 patients with systemic lupus erythematosus (SLE), 34 patients with silicone breast implants, and 30 patients with chronic fatigue syndrome (CFS) were studied for squalene antibodies. The study reports that a blinded test of serum samples found antibodies to squalene in more than 95 percent of 38 veterans deployed to the Gulf War who developed chronic illness symptoms; in all of the 6 veterans not deployed to the Gulf War who developed chronic illness symptoms; and in none of 12 veterans deployed to the Gulf War who were healthy. In an unblinded test, the study reported antibody reactivity to squalene in 5 percent of blood donors, 10 percent of patients with SLE, 10 percent of patients with silicone breast implants, and 15 percent of patients with CFS.

This study has several shortcomings. The subjects were self-selected, rather than being chosen at random from a larger sample, which can introduce substantial selection bias and does not allow inferences to the broader population of Gulf War veterans. Sample sizes were small, and the study may suffer from misclassification errors since the group of Gulf War veterans categorized as healthy ($n = 12$) was not devoid of individuals with serious symptoms (1 had fibromyalgia, 1 had thyroid disease, 3 had memory loss, and 4 had chronic fatigue). Further, the report provides inadequate evidence that the assay is able to accurately detect antibodies to squalene. Many of the methods used in the study are not described; as a result it is not possible to fully assess the study's methodology or to reproduce the assay. The study did not attempt to demonstrate that the substance giving the positive response in the assay was found in the immunoglobulin G (IgG) fraction of serum where antibodies are found. Further, the authors did not show that the assay was specific to squalene. To prove the specificity of the assay, the investigators would have had to show inhibition, in a dose-response manner, with squalene and no inhibition with other substances, as is seen in most reports of new enzyme-linked immunosorbent assays (ELISAs).

The committee does not regard this study as providing evidence that the investigators have successfully measured antibodies to squalene.

Future Research Directions Regarding Squalene

As squalene continues to be investigated for a number of clinical uses, ongoing toxicity studies will provide the additional information that is needed about its toxicity, both in animals and in humans. It will be important to examine the relevance of animal studies because of species differences in the absorption of squalene and the susceptibility of certain strains of animals to squalene's effects. In considering future research directions, the committee focused on squalene's potential use as a vaccine adjuvant. Research questions that remain to be addressed include the following:

- What types of immune responses does exogenous squalene evoke?
- Does the immune response differ with the route of administration or entry (i.e., oral, cutaneous, intramuscular)?
- How does the response vary according to the dose of squalene?
- Is the presence of antibodies to squalene abnormal, and if so, what is their functional significance?
- Could antibodies to squalene represent the consequences of, rather than the cause of, a pathological process?

CONCLUSIONS

The committee felt it would be helpful to the reader to restate the conclusions from this chapter. The conclusions listed below are identical to those made at the end of the respective sections of this chapter.

Anthrax Vaccine

There is a paucity of published peer-reviewed literature on the safety of the anthrax vaccine. The committee located only one randomized peer-reviewed study of the type of anthrax vaccine used in the United States (Brachman et al., 1962). However, the formulation of the vaccine used in that study differs somewhat from the vaccine given to Gulf War veterans (and currently in use). The Brachman study (and other early experimental studies) found transient local and systemic effects (primarily erythema, edema, induration) of the anthrax vaccine. There was no long-term monitoring for adverse outcomes. The committee did not compare the incidence of transient effects with other vaccines.

Studies of the anthrax vaccine have not used active surveillance to systematically evaluate long-term health outcomes. This situation is unfortunately typi-

cal for all but a few vaccines. The committee strongly encourages active monitoring to evaluate the long-term safety of the anthrax vaccine.

To date, published studies have reported no significant adverse effects of the vaccine, but the literature is limited to a few short-term studies. Reviewing the large body of results that have not yet been published may enable more definitive conclusions about the vaccine's safety. The committee strongly urges the investigators conducting studies on the safety of the anthrax vaccine to submit their results to peer-reviewed scientific journals for publication.

The committee's findings are best regarded as an early step in the complex process of understanding the vaccine's safety, which began with the vaccine's licensure in 1970 and the 1985 FDA advisory panel finding that categorized the anthrax vaccine as safe and effective. Active long-term monitoring of large populations will provide further information for documenting the relative safety of the anthrax vaccine.

The committee concludes that there is sufficient evidence of an association between anthrax vaccination and transient acute local and systemic effects (e.g., redness, swelling, fever) typically associated with vaccination.

The committee concludes that there is inadequate/insufficient evidence to determine whether an association does or does not exist between anthrax vaccination and long-term adverse health effects.

The latter finding means that the evidence reviewed by the committee is of insufficient quality, consistency, or statistical power to permit a conclusion regarding the presence or absence of an association between the vaccine and a health outcome in humans.

Botulinum Toxoid

Studies have noted transient local and systemic effects of the botulinum toxoid vaccine. However, studies of the botulinum toxoid vaccine have not used active surveillance to systematically evaluate long-term health outcomes. This situation is unfortunately typical for all but a few vaccines.

The committee concludes that there is sufficient evidence of an association between botulinum toxoid vaccination and transient acute local and systemic effects (e.g., redness, swelling, fever) typically associated with vaccination.

The committee concludes that there is inadequate/insufficient evidence to determine whether an association does or does not exist between botulinum toxoid vaccination and long-term adverse health effects.

The latter finding means that the evidence reviewed by the committee is of insufficient quality, consistency, or statistical power to permit a conclusion regarding the presence or absence of an association between the vaccine and a health outcome in humans.

Multiple Vaccinations

Certain multiple vaccination regimens can lead to suboptimal antibody responses, but there is little evidence, largely because of a lack of active monitoring, of other adverse clinical or laboratory consequences beyond the transient local and systemic effects seen frequently with any vaccination.

No long-term identifiable clinical sequelae attributable to intense long-term immunization occurred in the Fort Detrick cohort. There was some evidence of a chronic inflammatory response, but these changes cannot necessarily be attributed to the vaccinations, since the workers studied were occupationally exposed to a number of virulent microbes. This series of longitudinal clinical studies also had several shortcomings. However, the studies are valuable because careful monitoring did not disclose any evidence of serious unexplained illness in a cohort that received a series of intense vaccination protocols over many years.

The U.K. Gulf War studies provide some limited evidence of an association between multiple vaccinations and long-term multisymptom outcomes, particularly for vaccinations given during deployment (Unwin et al., 1999; Hotopf et al., 2000). There are some limitations and confounding factors in these studies, and further research is needed.

The committee concludes that there is inadequate/insufficient evidence to determine whether an association does or does not exist between multiple vaccinations and long-term adverse health effects.

This finding means that the evidence reviewed by the committee is of insufficient quality, consistency, or statistical power to permit a conclusion regarding the presence or absence of an association between multiple vaccinations and health outcomes in humans.

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